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THE FATE OF PHENOTHIAZINE IN RABBITS

I. THE DETECTION OF A NEW CONJUGATE IN RABBITS' URINE AFTER THE FEEDING OF PHENOTHIAZINE[1]

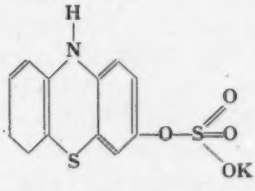
BY G. H. BENHAM[2]

Abstract

On feeding 5 gm. of phenothiazine to rabbits, approximately 50% of it is excreted in the urine as a colourless conjugate. This conjugate is readily hydrolysed by acids, giving the purple dye thionol and glucuronic acid. Determinations of both these entities suggest that the conjugate is composed of leuco thionol and glucuronic acid coupled together mole for mole.

Introduction

The widespread use of phenothiazine as an anthelmintic for sheep has disclosed the ability of that animal to oxidize and conjugate phenothiazine (17). This conjugate is colourless and soluble and is excreted in the urine. It was finally isolated by Collier (1), who proved it on analysis to be potassium leuco phenothiazone sulphate, with the following structural formula:



It was felt that some light might be shed on the fundamental mechanisms concerned in phenothiazine detoxication if further work were carried out, if possible on an animal smaller than the sheep.

The rabbit was selected as being a convenient animal with which to study this problem. Moreover, DeEds, Eddy, and Thomas (4, 18) have made some studies on the urine of rabbits after oral administration of phenothiazine. Thomas, McNaught, and DeEds have illustrated the low toxicity of phenothiazine to rabbits (19).

The earliest work on the application of phenothiazine for use in apple sprays in California was done by Eddy and DeEds (6). They reported that

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Contribution from the Institute of Parasitology, McGill University, Macdonald College, Que., with financial assistance from the National Research Council of Canada.

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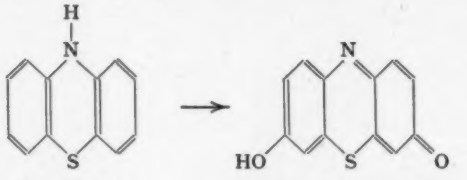
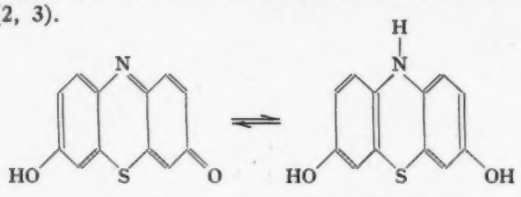
phenothiazine can be converted by chemical oxidation to 9-hydroxyphenothiazone:



Phenothiazine — 9-Hydroxyphenothiazone Thionol

This is commonly called thionol. The same workers subsequently showed that in the biological oxidation of phenothiazine by albino rats there was formed the same dye, thionol, which was passed in the urine (5). It was studied from the standpoint of its behaviour as part of an oxidation–reduction system, in which thionol and its reduced, colourless or "leuco" form are in equilibrium (2, 3).



Thionol — Leuco thionol

In studying the fate of phenothiazine in rats, rabbits, and man, Thomas, DeEds, and Eddy (18) state that the phenothiazine administered is excreted in three ways: (*a*) as the system thionol $\rightleftharpoons$ leuco thionol, (*b*) as phenothiazine, (*c*) as leuco thionol in some loose chemical combination, possibly with phenothiazine. In another paper these authors state that possibly conjugation occurs, but this study was not pursued any further.

This paper presents evidence for the elimination, by the rabbit, of a new conjugate after oral administration of phenothiazine. The mechanism appears to be entirely different from that observed in the sheep.

Experimental

Rabbits kept in suitable cages were given by mouth 5 gm. of phenothiazine in pellet form. The urine, when freshly voided, was the colour of normal urine, but it rapidly turned dark red. Spectrographic inspection of this colour indicated the bands characteristic of thionol rather than of phenothiazone (10). This is in line with the findings of DeEds and his collaborators. The oxidized phenothiazine leaves the body as the colourless leuco thionol, to be quickly oxidized on contact with air or treatment with mild oxidizing agents, to thionol. As such it is determined colorimetrically as follows:

The urine is suitably diluted, and one drop of potassium ferricyanide is added, to ensure complete oxidation of the leuco to the coloured form. This is recorded as free thionol (Table I).

TABLE I

DETERMINATION OF TOTAL, FREE, AND CONJUGATED THIONOL IN RABBITS' URINE AFTER ORAL ADMINISTRATION OF 5 GM. OF PHENOTHIAZINE

Rabbit No. 30. Samples taken on successive days

Total thionol, %	Total thionol, gm.	Free thionol, %	Conjugated thionol, %	Ratio conj. : free
1.180	1.300	0.014	1.166	82
0.680	0.850	0.015	0.665	45
0.271	0.402	0.008	0.269	35
0.103	0.123	0.007	0.096	14
0.023	0.023	—	—	—
0.009	0.014	—	—	—
	2.712 gm. thionol = 2.35 gm. phenothiazine (= 47% excretion)			
1.860	1.210	0.022	1.840	76
1.635	0.327	0.015	1.620	108
1.010	0.547	0.013	0.997	75
0.360	0.234	0.011	0.349	31
0.077	0.042	0.003	0.074	25
0.032	0.014	0.002	0.030	15
0.034	0.008	—	—	—
	2.392 gm. thionol = 2.08 gm. phenothiazine (= 42% excretion)			
2.090	1.150	0.0165	2.073	126
1.150	0.734	0.0130	1.137	89
0.736	0.552	0.0135	0.722	53
0.229	9.126	0.007	0.222	31
0.026	0.013	—	—	—
	2.575 gm. thionol = 2.24 gm. phenothiazine (= 45% excretion)			

Treatment of the urine with concentrated hydrochloric acid immediately throws down large quantities of a deep purple solid. This indicates that the urine contains a soluble conjugate, which is colourless, and which is broken down by acid hydrolysis. The dye thus obtained is identical with the dye present free in the urine, namely, thionol.

Suitable dilution of the urine, followed by complete acid hydrolysis gives a colour, which is read colorimetrically. It represents the total thionol originally present in the colourless conjugate, and the free form. Subtraction of the free thionol from the total thionol gives conjugated thionol (Table I). Dilutions were always adjusted so that readings of free and total thionol were in the same range on the colorimeter.

The findings reveal that the ratio of the conjugated to the free form is often as high as 100 to 1, denoting very efficient conjugation. Although the ratio decreases to 20 to 1 towards the end of the excreting period, the ability to conjugate seems to be fully regained at the beginning of a subsequent dose (Table I).

These results were very similar to those obtained with sheep, except that the dye eliminated by the sheep was phenothiazone rather than thionol. The results differed in that the conjugate from the sheep appeared to be more soluble and hence impossible to isolate at 0° C.

In order to determine the other partner in the conjugate, estimations were made of the total sulphate ion (Table II). The results show clearly that the sulphate does not increase on administration of phenothiazine (Fig. 1).

TABLE II

SULPHATE EXCRETED IN THE RABBIT, BEFORE AND AFTER DOSAGE WITH PHENOTHIAZINE

Rabbit No. 24. Samples taken on successive days

Conjugate, %	SO_4, %	SO_4, gm.
None	0.289	0.145
0.985	0.205	0.144
0.522	0.201	0.302
0.107	0.239	0.239
0.009	0.197	0.207
None	0.179	0.161
1.120	0.161	0.161
0.816	0.136	0.163
0.267	0.171	0.137
0.011	0.172	0.180
	Mean daily excretion	0.186 gm.

These preliminary determinations, representative of repeated dosages of both the same and different rabbits, are good evidence that detoxication of phenothiazine is carried out in the rabbit in an entirely different manner from that in the sheep.

On account of the well recognized ability of many animals, including the rabbit (8, 9, 22), to produce glucuronic acid for detoxication purposes, qualitative tests for glucuronic acid were carried out according to Tollens (20). Its presence was indicated in all urines, but the urine of animals dosed with phenothiazine gave particularly strong positive tests.

A qualitative method was sought because it was discovered that the total reducing substances could not satisfactorily be determined with Benedict's reagent, owing to interference with the end-point. Rough determinations showed, however, that, in urines in which the percentage of conjugated thionol was also high (Table III), increases in reducing substances were as

TABLE III

PERCENTAGE OF REDUCING SUBSTANCES (BENEDICT'S) (ON THE BASIS OF GLUCURONIC ACID, MOL. WT. 194) EXCRETED BY THE RABBIT NORMALLY AND AFTER DOSAGE WITH PHENOTHIAZINE

Rabbit No. 29. Samples taken on successive days

Conjugated thionol, %	Reducing substances, %	Reducing substances, mgm./day
1.17	1.01	1111
0.66	0.59	739
0.27	0.37	536
0.10	0.20	240
0.02	0.13	130
0.01	0.10	155
0.01	0.08	104
None	0.07	105
0.51	0.42	712
0.39	0.30	600
0.11	0.19	342
0.02	0.10	130
None	0.09	113

much as eightfold. Since Benedict's method is not specific for a particular reducing substance, an adaptation of Tollens' test was worked out in which glucuronic acid was specifically determined with an accuracy of about 1%.

Previous workers have attempted, with varying results, to adapt the Tollens' test to a quantitative procedure (7; 11; 13, pp. 436-440; 16; 21). It was discounted as an accurate method by some workers, but Mozolowski reported that the purple colour obtained after ether extraction was linear, provided the sample was sufficiently dilute with respect to glucuronic acid, but not otherwise (12).

The various methods were checked, and Mozolowski's findings were confirmed in every particular. Only two minor changes were made for convenience.

(*a*) 0.1% Aqueous naphthoresorcinol was used, instead of 0.2%. This gave a clearer colour, and less chance of precipitation on cooling.

(*b*) For extraction, 10 ml. of ether was used, rather than 15 ml.

The method then became as follows:—

Urine was diluted to such an extent that 2 ml. of the diluted urine contained 0.1 mgm. of glucuronic acid. Into three test-tubes were placed respectively 2, 1, and 0.5 ml. of this diluted urine; the second and third tubes were made up to 2 ml. with distilled water. Two millilitres of 0.1% aqueous naphthoresorcinol, in every case freshly prepared on the previous day, was added to each tube, followed by exactly 2 ml. of concentrated hydrochloric acid.

The tubes were transferred immediately to a vigorously boiling water-bath and left for exactly 30 min. After "freezing" the reaction in iced water for 10 min., the mixture, now of a steely gray colour if glucuronic acid is present, was quantitatively transferred to a 25 ml. glass-stoppered measuring cylinder;

the tube was rinsed into the cylinder with 2 ml. of 95% ethyl alcohol and two changes of exactly 5 ml. of ethyl ether. The cylinder was stoppered and well shaken for 30 sec. On allowing the solution to stand, a purple ether layer separated if glucuronic acid was present. This colour was found to be independent of the presence or absence of thionol, which is also purple. Five millilitres of the ether layer was removed and determined colorimetrically against standards treated in exactly the same way and simultaneously.

The standard glucuronic acid solutions were made by the following techniques, developed by Quick (14, 15).

Rabbits were fed either menthol or borneol, and the urine was collected. The glucuronate was isolated either as ammonium menthol glucuronate or as zinc borneol glucuronate; these salts are readily obtained in a pure state and lend themselves to the preparation of standard solutions.

Results

(1) Of 5 gm. of phenothiazine given to a rabbit, 45% may be excreted in the urine as a conjugate, most of it in the first 36 hr.

(2) The concentration of conjugated material sometimes exceeds 2%, especially when urine volume is low. This is higher than that obtained from the sheep. .

(3) This conjugate is much more soluble than that eliminated by the sheep. Quantities as high as 2% fail to precipitate out at 0° C., whereas cooling sheeps' urine from body temperature to 0° C. causes a voluminous precipitate of the conjugate in the form of fine yellow needles (Collier (1)).

(4) Even when the quantity of the conjugated thionol is as high as 2%, there is no increase in total sulphate excretion.

(5) Comparisons of the conjugated thionol with total glucuronic acid in the same urines were worked out in percentages (Table IV), in mgm. per day,

TABLE IV

PERCENTAGE OF CONJUGATED THIONOL AND TOTAL GLUCURONIC ACID IN RABBITS' URINE AFTER ORAL ADMINISTRATION OF PHENOTHIAZINE

Rabbit No. 24. Samples taken on successive days

Thionol in the conjugated form, %	Total glucuronic acid, %	Thionol in the conjugated form, %	Total glucuronic acid, %
1.28	0.91	None	0.20
0.30	0.28	None	0.06
0.20	0.38	1.24	0.96
0.05	0.22	1.60	1.03
0.03	0.25	0.65	0.43
0.01	0.17	0.15	0.18
None	0.15	0.12	0.07
None	0.09	1.02	0.67
None	0.18	0.12	0.27
None	0.21	0.01	0.27
None	0.17	0.01	0.20

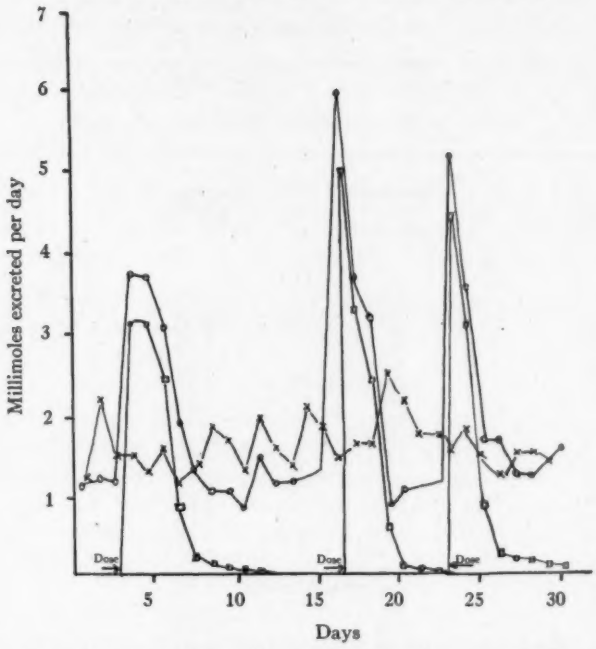
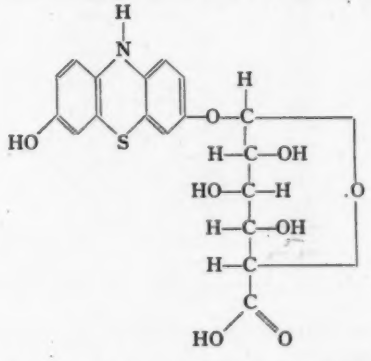


FIG. 1. *Daily excretion of conjugated thionol, glucuronic acid, and sulphate in rabbits after oral administration of phenothiazine.* □——□, *Millimoles of conjugated thionol;* ○——○, *millimoles of glucuronic acid;* ×——×, *millimoles of sulphate.*

and in millimoles per day (Table V). The figures for millimoles per day are shown graphically in Fig. 1. This is direct evidence that leuco thionol and glucuronic acid are present mole for mole. These two substances might, therefore, be linked together in some such way as the following:



It will be noted that the glucuronic acid excretion is higher, mole for mole, than that of the conjugated thionol. The average excess in millimoles per

TABLE V

EXCRETION OF CONJUGATED THIONOL AND TOTAL GLUCURONIC ACID BY THE RABBIT AFTER INTERMITTENT 5 GM. DOSES OF PHENOTHIAZINE

Rabbit No. 29. Samples taken on successive days

Conjugated thionol, mgm./day	Glucuronic acid, mgm./day	Conjugated thionol, millimoles/day	Glucuronic acid, millimoles/day
None	215	None	1.11
None	225	None	1.16
None	222	None	1.14
710	718	3.10	3.70
710	700	3.10	3.66
547	589	2.39	3.02
192	358	0.81	1.85
41	226	0.18	1.17
25	190	0.11	0.98
21	182	0.09	0.94
3	148	0.01	0.76
None	277	None	1.43
None	204	None	1.11
None	142	None	0.73
1140	1160	4.98	5.97
735	710	3.22	3.66
555	610	2.38	3.16
125	163	0.55	0.84
12	200	0.06	1.03
946	1000	4.20	5.15
694	692	3.04	3.57
179	318	0.78	1.65
46	319	0.20	1.64
24	103	0.11	0.53
26	300	0.12	1.55
None	159	None	0.82
None	334	None	1.72
None	100	None	0.52
None	200	None	1.04
None	109	None	0.56
None	206	None	1.06
None	218	None	1.13
None	255	None	1.32
None	322	None	1.71
None	264	None	1.36
416	370	1.82	1.92
1752	1660	7.65	8.57
241	468	1.05	2.05
146	450	0.64	1.96
92	350	0.40	1.53
11	235	0.05	1.21
1047	1350	4.55	6.90
650	885	2.84	4.60
120	533	0.53	2.74
27	282	0.12	1.45

day, as compared with glucuronic acid excretion in the normal urine from the same rabbits, is given below.

Within the limits of error in such an experiment, the results therefore show that, in addition to a certain basal excretion of glucuronic acid, the rabbit

Rabbit No.	Glucuronic acid			
	Number of determinations	Normal urine, millimoles/day, av.	Number of determinations	Excess glucuronic acid over thionol conjugate, millimoles/day
29	16	1.11	29	1.0
30	8	0.77	16	0.75
24	2	0.79	15	0.77

dosed with phenothiazine produces a conjugate that consists of thionol coupled mole for mole with glucuronic acid.

Conclusion

It seems that the rabbit utilizes a very large source of glucuronic acid for the purpose of conjugation with oxidized forms of phenothiazine. Attempts to isolate this conjugate have so far been unsuccessful, but further work is continuing to this end. Subsequent experiments will be carried out to establish the source of this large amount of glucuronic acid and the site of its formation.

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INFLUENCE OF SULPHONAMIDES ON FIBROBLASTS[1]

By Reta Anderson[2], J. H. Orr[3], and G. B. Reed[3]

Abstract

The toxicity of several sulphonamides for tissue cells has been tested by adding the compounds to tissue cultures of fibroblasts (guinea pig heart tissue in guinea pig serum). Supersaturated solutions of sulphadiazine or sulphapyrazine, in serum, have no influence on the growth of fibroblasts. Sulphathiazole, N^1-benzoyl-sulphanilamide, or sulphanilamide in similar concentrations inhibit fibroblast growth. The above-mentioned five sulphonamides in concentrations up to 100 mgm. per 100 ml. have no apparent influence on fibroblast growth. In contrast, azochloramide in concentrations of 5 to 10 mgm. per 100 ml. inhibit growth.

Jacoby, Medawar, and Willmer (2) found that fibroblasts will grow in tissue cultures in the presence of large concentrations of sulphonamides. Similar results were obtained by Reed, Orr, and Anderson (7). It was shown that normal growth of these cells occurs in serum containing sulphathiazole in concentrations of 80 to 100 mgm. per 100 ml. In higher concentrations of sulphathiazole, growth was inhibited, but if after several days in saturated solution of sulphathiazole in serum the cells are transferred to normal serum, characteristic growth occurs.

In the present paper a more extensive series of experiments are reported in which the action of several sulphonamides is compared and contrasted with the effect of azochloramide, both alone and as a supplement to sulphonamides. The object of the investigation is to assess the possible toxic action of the drugs in local chemotherapy.

Method

The procedure followed was that of des Ligneris (3) and Lumsden (4) in which fibroblasts were grown in serum. Serum was collected from guinea pigs and stored in capillary tubes for use as described by Lumsden. Fresh serum was necessary for drug dilutions every three or four days, as serum showed deterioration in growth-promoting quality after this time.

Accurate measurements of sulphonamide drug concentrations were made by sterilizing small measured amounts at 145° C. for one hour, then dissolving in sterile water to make concentrations from 5 mgm. per 100 ml. to supersaturation. Solutions were adjusted to pH 7.2 and small amounts, usually 2 cc., dispensed in sterile tubes. After the solution was evaporated to dryness, a corresponding amount of serum was added. The instability of azochloramide in the presence of heat and light prevented sterilization of an aqueous solution. This drug containing buffer-salt mixture was weighed aseptically in amounts required to produce concentrations of 100 mgm. per 100 ml.

[1] *Manuscript received February 21, 1945.*

Contribution from the Department of Bacteriology, Queen's University, Kingston, Ont., with financial assistance from the National Research Council of Canada.

[2] *Instructor.*

[3] *Professor.*

The drug was then dissolved in normal guinea pig serum to make concentrations of 100 mgm. per 100 ml., and, from this, serial dilutions were prepared in sterile, normal, guinea pig serum.

Heart muscle (auricle) of young guinea pigs was used as a source of fibroblasts. Minute fragments of this tissue were placed in drops of normal serum, or serum containing the material under tests, on cover glasses. These were immediately sealed on depression slides. At daily intervals the cover glasses were lifted and the culture solutions renewed.

As a test of the toxicity of an agent a number of cultures of fibroblasts were made up at the same time in normal serum and in serum containing the agent under test. These were observed for a period of seven days and the percentage of cultures showing normal growth determined. The explants of tissue were then washed with normal serum and reincubated for a further seven days in normal serum. Fig. 1 shows photomicrographs of a seven-day culture in serum containing sulphathiazole in a concentration of 50 mgm. per 100 ml.

Sulphonamides

A comparison has been made of the growth of fibroblasts in six sulphonamides of varying concentrations. The results are summarized in Table I.

TABLE I

INFLUENCE OF SULPHONAMIDES ON THE GROWTH OF FIBROBLASTS

Drug	No. of cultures	Percentage of cultures showing normal growth of fibroblasts							
		Drug present					Drug removed		
		1 Day	2 Days	3 Days	7 Days	14 Days	1 Day	3 Days	7 Days
Sulphathiazole									
Saturated	75	3	—	3	4	—	25	79	93
100 mgm./100 ml.	10	0	—	40	60	—	80	100	100
50 mgm./100 ml.	36	14	—	86	94	—	—	—	—
5 mgm./100 ml.	57	12	—	70	97	—	—	—	—
Nil	110	10	—	82	94	—	—	—	—
Sulphadiazine									
Saturated	40	10	—	55	65	95	—	—	—
Nil	20	25	—	90	100	100	—	—	—
Sulphapyrazine									
Saturated	25	8	—	48	88	92	—	—	—
Nil	20	10	—	40	85	95	—	—	—
N¹-benzoyl-sulphanilamide									
Saturated	40	—	0	0	0	—	0	0	0
100 mgm./100 ml.	30	—	43	60	96	96	—	—	—
10 mgm./100 ml.	10	—	40	30	90	90	—	—	—
Nil	20	—	55	30	80	90	—	—	—
Sulphanilamide									
Saturated	20	—	0	0	0	—	0	0	0
100 mgm./100 ml.	50	—	0	48	84	86	—	—	—
50 mgm./100 ml.	30	—	27	73	93	93	—	—	—
10 mgm./100 ml.	10	—	40	70	90	90	—	—	—
Nil	20	—	30	45	85	85	—	—	—

Sulphathiazole. As indicated in Table I, sulphathiazole up to 50 mgm. per 100 ml. in serum had no inhibitory effect on the growth of fibroblasts. At 100 mgm. per 100 ml., growth is definitely retarded, and in saturated solution growth fails to occur except in the occasional culture. However, when these tissue masses were transferred to normal serum, characteristic growth occurred.

Sulphanilamide and N^1-benzoyl-sulphanilamide. These compounds up to 100 mgm. per 100 ml. in serum have no retarding effect on the growth of fibroblasts. In saturated solutions, however, no growth occurs and the cells fail to recover on transfer to normal serum.

Sulphadiazine and sulphapyrazine. In saturated solutions in serum, which is well over 100 mgm. per 100 ml., these two compounds exert no detectable retarding effect on the growth of fibroblasts.

In view of the well known distribution of sulphonamide inhibitors in tissues, MacLeod (5), it seemed desirable to make some estimate of the amount of inhibitor substance in cultures of growing fibroblasts. The small amount of material in the tissue cultures was tested for inhibitors by the methods of Bliss and Long (1), and Reed, Orr, and Reed (8).

TABLE II

SULPHONAMIDE INHIBITOR CONCENTRATION IN SERUM AND IN GROWING CULTURES OF FIBROBLASTS

Synthetic medium for *E. coli*, with additions	Growth of *E. coli*	
	In 24 hr.	In 48 hr.
Synthetic medium, 10% normal serum	+	+
Synthetic medium with sulphanilamide (10 mgm. per 100 ml.)	−	−
Synthetic medium with 10% serum and sulphanilamide (10 mgm. per 100 ml.)	−	+
Synthetic medium with fluid from tissue culture originally containing 10% serum and sulphanilamide (10 mgm. per 100 ml.)	−	+

It is apparent from results shown in Table II that sulphanilamide in a concentration of 10 mgm. per 100 ml., in synthetic medium, prevents the growth of *Escherichia coli;* the same mixture of sulphanilamide with the addition of 10% of serum or serum in which fibroblasts have grown retards but does not prevent the bacterial growth. This appears to indicate that sulphonamide inhibitors are present in the serum or produced by the growing fibroblasts in sufficient concentration to partly inhibit the bacteriostatic action of sulphanilamide in a concentration of 10 mgm. per 100 ml. Where the drug is added to provide concentrations of 50 to 100 mgm. per 100 ml. there must be a large residue of material still bacteriostatic.

PLATE I

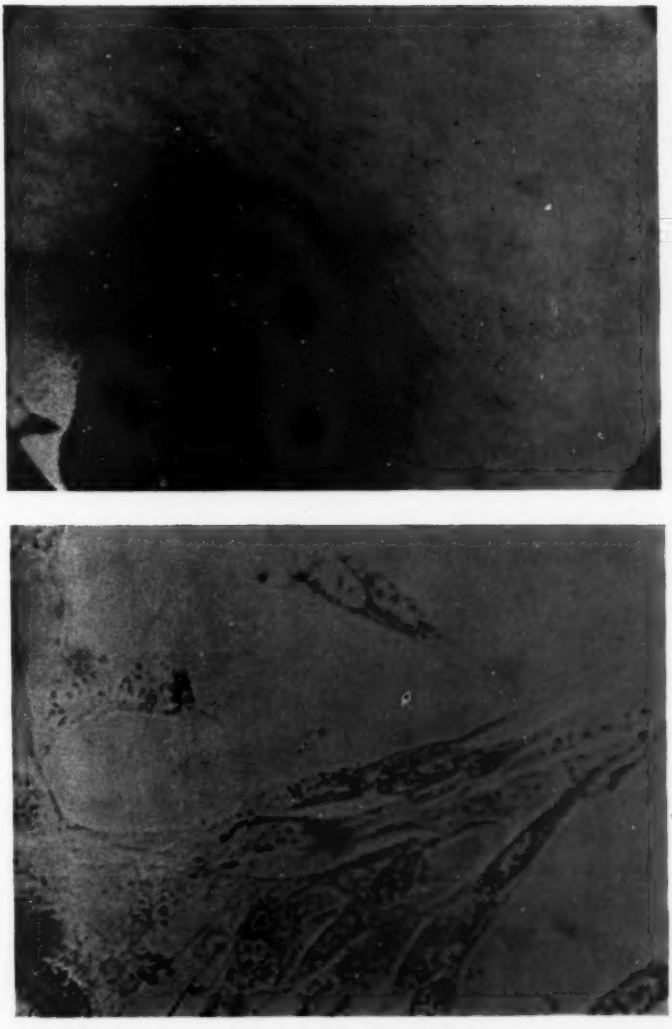

FIG. 1. *Photomicrographs of fibroblasts growing in tissue cultures in a serum medium containing sulphathiazole in a concentration of 50 mgm. per 100 ml. Low and high power views of the same culture.*

Azochloramide

This is a relatively stable chlorine compound which retains much of its bacteriostatic or bactericidal properties in the presence of organic matter.

As indicated in Table III, azochloramide in a concentration of 1 mgm. per 100 ml. permits normal growth of fibroblasts and 2 to 4 mgm. per 100 ml. is only very slightly toxic. Greater concentrations are, however, definitely

TABLE III

GROWTH OF FIBROBLASTS IN SERUM CONTAINING AZOCHLORAMIDE

Azochloramide, mgm. per 100 ml.	No. of cultures	Percentage of cultures showing normal fibroblast growth			
		Azochloramide present			
		1 Day	3 Days	7 Days	14 Days
25	10	0	0	0	—
10	20	0	0	0	—
5	20	0	30	60	80
4	20	5	30	70	90
2	20	5	35	65	70
1	10	20	60	100	100
Nil	30	23	70	90	90

toxic; 5 mgm. per 100 ml. inhibits growth but some cultures recover on transfer to serum, while still higher concentrations kill the cells. This is the same order of toxicity for fibroblasts as shown by Schmidt *et al.* (10) with a different technique.

It is therefore apparent that in contrast to sulphonamides this compound is highly toxic to fibroblasts.

Sulphonamides and Azochloramide

Several authors have indicated a synergistic action of sulphonamides and azochloramide. Schmelkes and Wyss (9) found that the *in vitro* growth of *E. coli* is inhibited by a mixture of sulphanilamide and azochloramide at concentrations that singly fail to inhibit growth. Neter (6) has shown a similar action on haemolytic *Streptococcus*, *Staphylococcus*, and *Pneumococcus*. They, however, failed to find evidence of increase in local chemotherapeutic activity of sulphanilamide by adding azochloramide.

The growth of fibroblasts has been examined in serum containing sulphadiazine or sulphapyrazine alone, and azochloramide alone or in serum with the sulphonamide and azochloramide, Table IV. It is apparent from the table that concentrations of sulphonamide alone or azochloramide alone that are not toxic to fibroblasts are not toxic in combination. There is therefore no indication of a synergistic action of the two compounds on fibroblasts.

TABLE IV

GROWTH OF FIBROBLASTS IN SERUM CONTAINING AZOCHLORAMIDE, SULPHONAMIDES AND COMBINATIONS OF THE TWO COMPOUNDS

Drug	Percentage of cultures showing normal growth of fibroblasts			
	1 Day	5 Days	10 Days	14 Days
Azochloramide only (4 mgm./100 ml.)	10	60	90	100
Sulphadiazine only (saturated)	20	100	100	100
Sulphapyrazine only (saturated)	0	70	100	100
Sulphadiazine (saturated) plus azochloramide (4 mgm./100 ml.)	0	90	100	100
Sulphapyrazine (saturated) plus azochloramide (4 mgm./100 ml.)	0	70	100	100
Nil	30	100	100	100

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N^1-BENZOYL-SULPHANILAMIDE IN EXPERIMENTAL GAS GANGRENE[1]

By G. B. Reed[2] and J. H. Orr[2]

Abstract

It is shown that in regard to gas gangrene *Clostridium* the *in vitro* bacteriostatic action of N^1-benzoyl-sulphanilamide is similar to that of sulphathiazole and sulphadiazine. In local treatment of experimental gas gangrene in guinea pigs, N^1-benzoyl-sulphanilamide is equal to sulphathiazole in retarding infection with *C. welchii*, *C. novyi*, or *C. septicum*. It is slightly superior to sulphathiazole in retarding *C. sordellii* infections.

Siebenmann and Schnitzer (5) recently described the preparation of a series of *p*-nitrobenzoyl and related compounds. Among these N^1-benzoyl-sulphanilamide exhibited marked chemotherapeutic activity against pneumococci.

Through the kindness of Dr. Siebenmann and Dr. D. T. Fraser, Connaught Laboratories, Toronto, the writers have received a generous supply of N^1-benzoyl-sulphanilamide which has been tested against the gas gangrene group of anaerobes both *in vitro* and in local application to experimental gas gangrene wounds in guinea pigs.

In vitro Action on Gas Gangrene Organisms

In a previous report, Reed, Orr, and Reed (4), it was shown that all sulphonamides tested up to that time have a comparatively slight inhibitory action on the test-tube growth of *C. welchii* and several non-pathogenic or slightly pathogenic members of the genus. Concentrations of the order of 100 to 200 mgm. per 100 ml. in the culture fluid are necessary to inhibit growth. In contrast, the growth of *C. novyi* was shown to be inhibited by 10 to 20 mgm. per 100 ml. and the growth of *C. septicum* and *C. sordellii* was inhibited by most sulphonamides in concentrations of 1 to 2 mgm. per 100 ml. This wide difference in the *in vitro* growth-inhibitory action was shown to be due to a difference in the production of sulphonamide inhibiting substances by the different species. *C. welchii* was shown to produce the order of 30 times more sulphonamide inhibitor than *C. sordellii* when grown under similar conditions in culture fluid.

The *in vitro* inhibitory action of N^1-benzoyl-sulphanilamide in comparison with sulphathiazole on the growth of four species of gas gangrene clostridia, when tested under conditions similar to those previously reported, is summarized in Table I. It will be observed that the inhibitory action of the two sulphonamides is almost identical.

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Contribution from the Department of Bacteriology, Queen's University, Kingston, Ont., with financial assistance from the National Research Council of Canada.

[2] *Professor.*

TABLE I

In vitro INHIBITION OF GROWTH OF SEVERAL SPECIES OF *Clostridium* BY N^1-BENZOYL-SULPHANILAMIDE IN CONSTRAST TO SULPHATHIAZOLE

	Smallest concentration to inhibit growth	
	N^1-benzoyl-sulphanilamide, mgm./100 ml.	Sulphathiazole, mgm./100 ml.
C. welchii		
1 Day	10	10
5 Days	100	200
C. novyi		
1 Day	2	2
5 Days	20	10
C. septicum		
1 Day	1	1
5 Days	2	3
C. sordellii		
1 Day	1	1
5 Days	2	2

Local Treatment of Gas Gangrene in Guinea Pigs

In previous reports, Reed, and Orr (2, 3), it was shown that when applied locally as a dry powder to the infected wounds several sulphonamides are highly effective in the control of experimental gas gangrene in guinea pigs.

The procedure followed in testing the local chemotherapeutic effects of N^1-benzoyl-sulphanilamide was the same as in earlier experiments with other sulphonamides. The thighs of 300- to 350-gm. guinea pigs were shaved and an incision made deep into the musculature. A fragment of muscle was excised and placed deep down in the incision along with 0.1 gm. of finely ground *sterile* garden soil. One-twentieth cubic centimetre of 1-1000 dilution of a 24-hr. broth culture of the anaerobe to be tested was then introduced and the wound tightly closed with two rows of continuous sutures, one in the muscle and one in the skin.

In the present experiments 0.015 gm. of N^1-benzoyl-sulphanilamide or a similar amount of sulphathiazole was introduced as a dry powder just before the wound was closed.

The results are summarized in Table II. It will be observed that a single local application, introduced into the wound immediately after the introduction of the infecting organisms, is highly effective against *C. welchii*, *C. novyi*, and *C. septicum* infections and only slightly less effective with *C. sordellii* infections.

Comparison of these data on N^1-benzoyl-sulphanilamide with previously reported results, Reed and Orr (3), with several sulphonamides in a much larger group of guinea pigs with similarly induced gas gangrene is summarized

TABLE II

ACTION OF THE LOCAL APPLICATION OF N¹-BENZOYL-SULPHANILAMIDE ON EXPERIMENTAL GAS GANGRENE IN GUINEA PIGS

No. animals	Medication with N¹-benzoyl-sulphanilamide, gm.	Per cent recovered	Per cent died	Average survival time, hr.
C. welchii, *WX*				
10	Nil	0	100	28
20	0.015	90	10	220
C. novyi, 277				
10	Nil	0	100	49
10	0.015	90	10	139
C. septicum, 14				
10	Nil	0	100	61
10	0.015	100	0	—
C. sordellii, 213				
10	Nil	0	100	32
16	0.015	83	17	82

in Table III. It is apparent from the table that recovery rates from *C. welchii*, *C. novyi*, and *C. septicum* experimental gas gangrene are essentially the same with a single local application of N¹-benzoyl-sulphanilamide, sulphathiazole, or sulphadiazine.

TABLE III

COMPARISON OF RECOVERY OF GUINEA PIGS GIVEN ONE LOCAL DOSE OF SULPHONAMIDE FOLLOWING INFECTION

Experimental gas gangrene infection	Percentage of guinea pigs recovering after one local treatment with:				
	Sulphanil-amide	Sulpha-pyridine	Sulpha-thiazole	Sulpha-diazine	N¹-benzoyl-sulphanilamide
C. welchii	40	73	97	80	90
C. novyi	24	24	85	40	90
C. septicum	25	50	87	100	100
C. sordellii	40	60	30	28	83

Results, as previously reported, with *C. sordellii* gas gangrene have been more irregular than those obtained with other gas gangrene infections. For the most part sulphonamide therapy has been ineffective. As indicated in Table II, N¹-benzoyl-sulphanilamide gave an 83% recovery rate, or almost as high as that obtained with the other gas infections. A similar group of guinea pigs infected at the same time from the same culture of *C. sordellii* and treated locally with 0.015 gm. of sulphathiazole gave a 30% recovery rate. These data, together with the previous reports, Reed and Orr (3), indicate that N¹-benzoyl-sulphanilamide is more effective as a local chemotherapeutic

agent in *C. sordellii* infections than any chemotherapeutic drug so far tested. It should be noted, however, that the balance of evidence suggests that *C. sordellii* is a relatively rare organism in human gas gangrene.

Influence on the Growth of Fibroblasts

In an accompanying paper, Anderson, Orr, and Reed (1), it is shown that fibroblasts grow at a normal rate in serum containing N^1-benzoyl-sulphanilamide in concentrations up to 100 mgm. per 100 ml.

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STUDIES ON EXPERIMENTAL SHOCK IN DOGS[1]

By J. I. Hamilton[2] and R. E. Haist[3]

Abstract

A method is described for producing shock in dogs by application of pressure cuffs to the hind legs. The blood pressure rose when the cuffs were applied and remained high during the period of application. When the cuffs were removed the blood pressure fell. The average survival time of 34 dogs was three hours, 20 min. Among the changes noted were an increase in haematocrit value, a rise in the lactic acid concentration in the femoral vein, and a rapid decrease in the oxygen content of jugular vein blood with maintenance of the arterial oxygen content until shortly before death. The plasma protein concentration usually rose but was variable.

The intermittent application of pressure cuffs, using 20 min. periods of application with five minute release intervals, produced no serious effects, but with 40 min. periods of application signs of shock developed. When haemorrhage preceded the application of cuffs for the 20 min. periods, recovery usually occurred but when the haemorrhage preceded the 40 min. periods, death resulted.

Re-application of narrow band tourniquets to the injured legs of the shocked dogs produced an increase in blood pressure and an alleviation of their condition. With compression of one leg for 10 hr. the changes in total limb volume and haematocrit value were similar to those obtained when both legs were compressed for five hours. The dogs with one cuff applied for 10 hr. survived while those with two cuffs applied for five hours did not. It is felt that fluid loss alone does not account for all the changes observed in these experiments.

In order to study the problem of shock experimentally, some uniform method of producing shock must be available. The method used in these experiments is a modification of tourniquet procedures and was first described by the authors in a report to the National Research Council in 1941. The method was found to produce reasonably consistent results. Some of the changes occurring in dogs shocked by this procedure will be reported in this paper.

Materials and Methods

Dogs were anaesthetized with sodium pentobarbital given intravenously. Standard blood pressure cuffs were wrapped securely around the hind limbs as high up as possible in order to include the greatest possible mass of muscle. Cotton gauze padding placed around the knees helped to keep the cuffs in position and allowed a more uniform application of pressure (Fig. 1). The pressure in the cuffs was kept above 230 mm. mercury for five to six hours, unless otherwise stated. A warming cradle was placed over the hind limbs and removed at the same time as the cuffs. After removal of the pressure cuffs the animals became shocked and died in from one to eight hours.

Control dogs were treated in the same way as the test animals except that no pressure cuffs were applied. It should be pointed out, however, that control animals required repeated doses of the anaesthetic throughout the

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entire experiment, whereas, after removal of the cuffs, the shocked animals seldom required any additional anaesthetic.

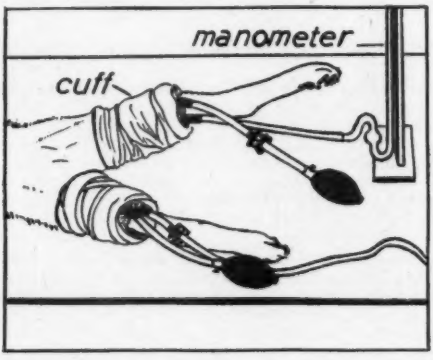


FIG. 1. *Method of producing shock. The pressure cuffs and padding are in place. The warming cover is not shown.*

In acute experiments the carotid artery was cannulated and continuous blood pressure records were obtained. Heparinized blood was used for haematocrit estimations. All samples were centrifuged in 15 cc. graduated tubes for 40 min. at 3000 r.p.m.

The swelling of the legs was measured by the volume displacement method of Solandt and Best (6). The measurement of limb volume was made before the cuffs were applied and was repeated after shock was well established.

Female dogs were used for the study of urinary changes. They were catheterized before removal of the cuffs. Urine for analysis was collected before and after removal of the cuffs and for several days following the test. In survival experiments, a cuff was applied for 5 or 10 hr. to one leg.

Plasma protein and non-protein nitrogen concentrations were determined by a micro Kjeldahl technique. Lactic acid was measured by the method of Barker and Summerson (1). The oxygen content of blood was estimated by the method of Roughton and Scholander (5).

Experimental Results

Survival

The survival time of 34 dogs shocked by this method (two cuffs) ranged from one to eight hours. The average was three hours and 20 min. The data are given in Fig. 2. One dog recovered and lived for several days.

Blood Pressure

Following the application of the pressure cuffs, the carotid blood pressure usually increased (Table I). As long as the pressure in the cuffs was maintained, shock did not develop. When the cuffs were removed the blood pressure quickly fell. Subsequently, the pressure sometimes rose again to the

previous level and then more slowly declined. The pressure frequently was maintained between 60 and 90 mm. mercury for from one to five hours before

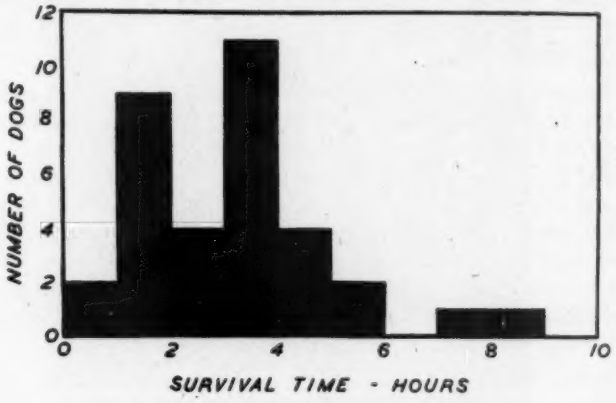


FIG. 2. *Survival times for 34 shocked dogs.*

TABLE I

THE EFFECT ON BLOOD PRESSURE OF THE APPLICATION OF BLOOD PRESSURE CUFFS (AT 240 MM. MERCURY) TO THE HIND LEGS OF DOGS

Blood pressure, mm. mercury					
Before application of pressure cuffs	30 Min. after application	Increase	Before application of pressure cuffs	30 Min. after application	Increase
151	164	13	134	158	24
151	166	15	137	160	23
170	200	30	137	166	29
184	210	26	125	125	0
108	186	78	140	144	4
160	176	16	152	230	78
134	164	30	124	156	32
136	154	18	116	174	48
130	158	28	134	154	20
108	154	46	111	126	15
174	212	38			
Average increase 30 mm. mercury					

death. Fig. 3 shows portions of a typical blood pressure record (see also Fig. 10) and Fig. 4 includes the blood pressure changes in 20 shocked dogs. The changes in blood pressure in general were similar to those reported by Wilson and Roome (7) to follow removal of tourniquets, though the survival times were much longer in their experiments.

In two out of 15 controls the blood pressure fell gradually to shock levels, possibly owing to an excess of the anaesthetic. Because no additional

anaesthetic was required after the cuffs were released, it was felt that each experimental animal was its own control since the constancy of the blood pressure prior to removal of the cuffs provided a very good index of the influence of the anaesthetic.

Haematocrit Values

The haematocrit values rose rapidly after removal of the cuffs, reaching an average final value of 70 (50 to 81% cells). There was no appreciable increase in the haematocrit values in control animals. In the shocked animals, the femoral vein blood showed a small but consistent elevation over that of the femoral artery or jugular vein. Fourteen sets of haematocrit estimations on shocked animals gave an average of 66.1 for jugular vein, 69.7 for femoral vein, and 66.5 for femoral artery. The femoral vein values were tested statistically and found to be significantly higher. Seven control sets averaged 53.2 for jugular vein, 52.3 for femoral vein, and 52.4 for femoral artery. The data are given in Table II.

Limb Volumes and Fluid Loss

After the removal of the cuffs, the limbs became swollen rather rapidly, the major swelling being in the region that had been underneath the cuffs. The changes are shown in Table III. The increase in volume of the two hind legs in the shocked animals is expressed as a percentage of the body weight. The average fluid loss for dogs was 2.35% of the body weight or 28.8% of the calculated blood volume, assuming the latter to be 8% of the body weight. The fluid lost into the injured area did not contain a significant number of red cells. At autopsy, only a clear oedema fluid could be seen. If the blood volume is taken to be 8% of the body weight and the plasma volume is estimated from this, using the haematocrit readings, the reduction in blood fluid in the shocked animals is calculated to be 54% of the plasma volume on the average. There is little indication, except in isolated cases, that any significant amounts of fluid are lost from the blood vessels elsewhere than in the damaged limbs. The loss of fluid estimated from the haematocrit changes agrees moderately well with the observed increases in limb volume. The average observed increase in limb volume was 265 cc. The average estimated fluid loss based on the haematocrit changes was 224 cc. for 13 dogs. Since any release of cell-rich blood from blood reservoirs would make this value higher, it seems that some fluid is withdrawn from other tissues in the shocked animal and enters the blood.

Plasma Proteins

In five control animals anaesthetized for long periods, the plasma protein concentration did not vary appreciably. In most of the shocked animals the plasma protein concentration increased, though this change was variable. Of 13 dogs, one showed a decrease, two no change, five an increase of 6 to 20%, and five an increase of over 20%. In those animals showing an increase in plasma protein, a relatively greater amount of fluid than protein would seem to have left the blood vessels.

PLATE I

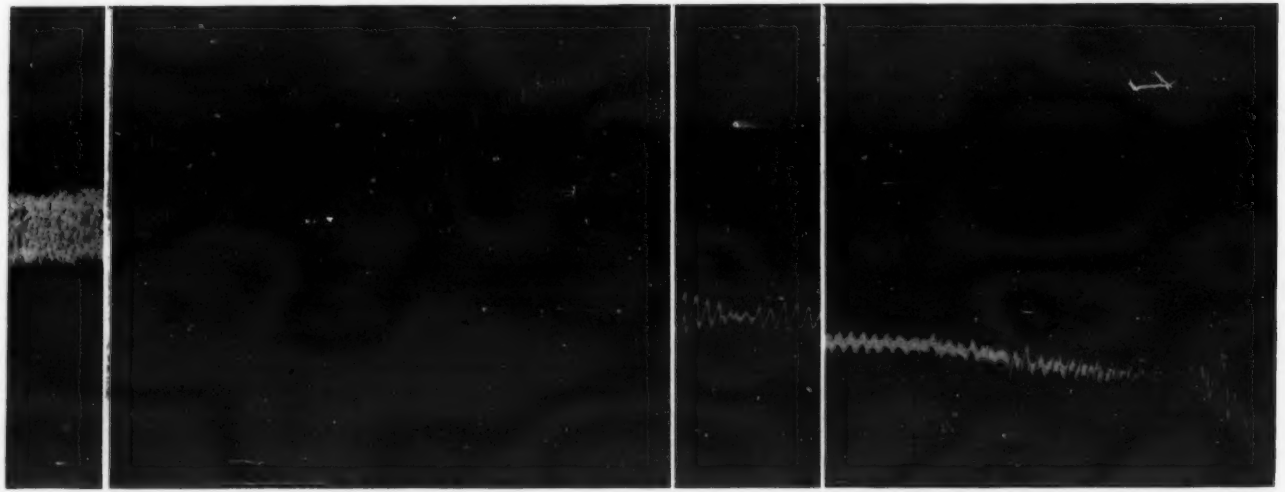


FIG. 3. *Record of carotid blood pressure in a shocked dog. The mean pressure at the start of this tracing is 116 mm. mercury. This shows the rise in pressure after the application of the cuffs and the changes following removal.*

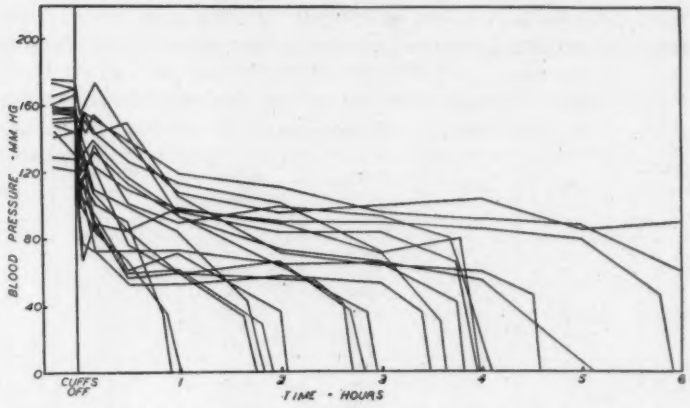


FIG. 4. *The blood pressure changes in 20 shocked dogs.*

TABLE II

ARTERIOVENOUS DIFFERENCES IN HAEMATOCRITS

	Minutes after removal of cuffs					
	30 – 120*			120 – Death*		
	Haematocrits			Haematocrits		
	Jug. vein	Fem. vein	Fem. art.	Jug. vein	Fem. vein	Fem. art.
Shocked dogs						
No.						
58	—	—	—	63.0	63.8	61.7
68	54.4	58.3	58.2	—	—	—
69	66.0	70.4	65.8	67.9	73.6	68.8
70	—	—	—	62.9	74.1	70.7
71	65.2	69.1	63.2	69.5	75.5	72.9
72	52.7	54.7	50.8	51.2	53.4	50.0
75	—	—	—	73.2	80.0	74.4
76	74.5	77.0	72.2	79.7	80.0	79.4
89	—	—	—	74.6	72.7	72.5
90	70.2	71.2	70.8	—	—	—
Control dogs						
No.						
73	50.7	46.7	50.4	50.7	48.2	49.5
80	50.0	50.0	47.9	46.8	48.5	45.7
94	—	—	—	59.6	58.7	59.6
95	54.8	55.9	55.4	60.0	58.3	58.1

* *For control dogs these times are minutes after the anaesthetic was given.*

TABLE III

CHANGES IN LIMB VOLUMES AFTER THE APPLICATION OF PRESSURE CUFFS TO BOTH HIND LEGS OF DOGS FOR FIVE HOURS

Body weight, kgm.	Survival time		Limb volumes, cc.		Change in limb volumes after shock, cc.	Change expressed as % body weight
	Hr.	Min.	Before shock	After shock		
6.5	3	30	595	640	45	0.69
15.0	3	45	1655	2030	375	2.50
7.65	4	54	660	800	140	1.83
14.4	3	0	1297	1510	213	1.48
9.2	3	55	885	1093	208	2.36
9.5	4	20	755	1005	250	3.46
14.0	3	55	1680	2065	385	2.75
16.2	1	20	1710	1950	240	1.48
9.0	3	41	630	875	245	3.78
6.0	3	53	455	670	225	3.75
5.9	2	0	520	620	100	1.70
13.6	4	5	1395	1860	465	3.42
19.5	8	5	1973	2530	557	2.86
9.3	3	23	1155	1210	55	0.59
15.6	7	28	1415	1915	535	3.43
10.2	5	5	1165	1390	225	2.21
15.0	2	0	1465	1725	260	1.73
14.2	4	0	1330	1770	440	3.12
10.2	4	46	965	1120	155	1.52
8.8	2	20	850	1160	310	3.52
19.1	3	0	1890	2180	290	1.52
15.5	3	25	1220	1540	320	2.06

Lactic Acid and Oxygen Data

Plasma lactic acid values were determined in a number of animals (Table IV). On the average the plasma lactic acid in shock increased from 35 mgm. to 117 mgm. %*.

TABLE IV

THE PLASMA LACTIC ACID CONCENTRATION IN SHOCKED DOGS (MGM. %)

Normal before cuffs applied	Just before cuffs removed	Late in shock
37	—	110
30	16	118
37	38	108
19	12	93
47	61	220
33	—	104
28	74	91
48	65	95
Average 35		117

* *The term mgm. % when applied to plasma or blood signifies mgm. per 100 cc.*

Samples taken from different situations before and after the cuffs were released indicated that there was an immediate outpouring of lactic acid from the anoxic tissue. Immediately after releasing the cuffs the lactic acid concentration in the blood from the femoral vein was very much higher than in the blood from the femoral artery or jugular vein. This difference tends to become less later in shock (Table V).

TABLE V

THE PLASMA LACTIC ACID CONCENTRATION (MGM.%) IN SAMPLES FROM DIFFERENT VESSELS OF SHOCKED DOGS

Time	Carotid artery	Jugular vein	Femoral artery	Femoral vein
Pre-anaesthetic		19		
When cuffs off		12	8	18
30 min. after		69	76	97
1 hr. 15 min. after		49	51	80
3 hr. 30 min. after (near death)		93	52	112
Pre-anaesthetic		28		
When cuffs off	60	74	56	79
5 min. after	103	94	105	193
1 hr. after	99	102	92	116
4 hr. after (near death)		91		107
Pre-anaesthetic		48		
When cuffs off	44	65	46	38
5 min. after	70	71	70	132
1 hr. after	90	99	99	129
3 hr. after (near death)	78	95	68	117
Pre-anaesthetic		25		
When cuffs off	62	54	63	71
5 min. after	58	65	50	92
1 hr. after	58	54	69	134
2 hr. after	68	81	72	97

Control animals under nembutal anaesthesia for long periods did not show an increase in plasma lactic acid concentration.

Arteriovenous oxygen differences were measured in blood going to and coming from the head. A sharp drop in jugular vein oxygen content was sometimes observed as early as five minutes after the pressure cuffs were released. This reduction progressed as the shock developed. The arterial oxygen saturation, however, was maintained in the normal range until within about five min. of death (Fig. 5).

The sudden fall in jugular vein oxygen content, despite the maintenance of a normal oxygen content in the blood from the carotid artery, probably reflects a reduction in the blood flow to the head. This occurs *quickly* following removal of the cuffs and progresses only slowly from that time on.

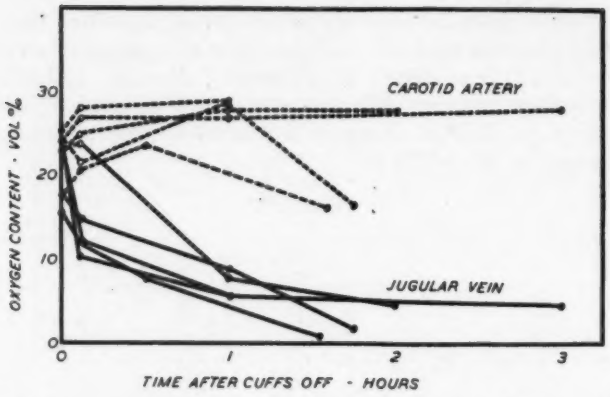


FIG. 5. *The oxygen content of blood from the carotid artery and jugular vein as shock progresses.*

*The Effect of Intermittent Compression**

In a series of dogs the pressure cuffs were applied for 20 min. periods at a pressure of 230 mm. mercury. They were released for five min. intervals and then re-applied. The sum of the periods of compression was five hours. There was a drop in blood pressure each time the cuffs were released and a return when the cuffs were re-applied, but the reduction was not progressive. Following the final removal of the cuffs the limbs did not swell appreciably and the animals survived (Fig. 6).

After removal of one-quarter of the estimated blood volume (2% of body weight) over a period of half an hour, the fall in blood pressure that followed release of the cuffs after each 20 min. period of application was greater, but four out of five animals recovered with no apparent ill effects (Fig. 7).

When the cuffs were applied for 40 min. periods with five minute release intervals, the immediate results were much the same as for the first experiment (20 min. periods), but one of these animals showed marked swelling and paralysis of one leg, and one did not live for 24 hr. (Fig. 8). The '40 min.' animals, which had one-quarter of their blood volume removed, all died within 24 hr., although in four cases out of five they had apparently recovered from the immediate effects of the haemorrhage. The blood pressures are given in Fig. 9. The results are collected in Table VI. It would seem, from this data, that periods of application of 20 min. or less, as carried out in this experiment, are not conducive to shock even when the blood volume is considerably reduced by previous bleeding, but that 40 min. periods of application may be followed by undesirable changes, which, after haemorrhage, frequently lead to death.

* *This study was undertaken at the request of Dr. W. B. Cannon, National Research Council, Washington.*

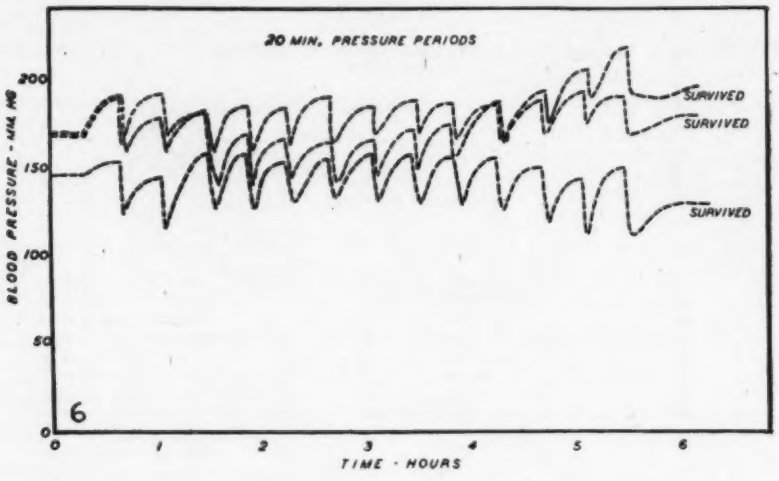
TABLE VI

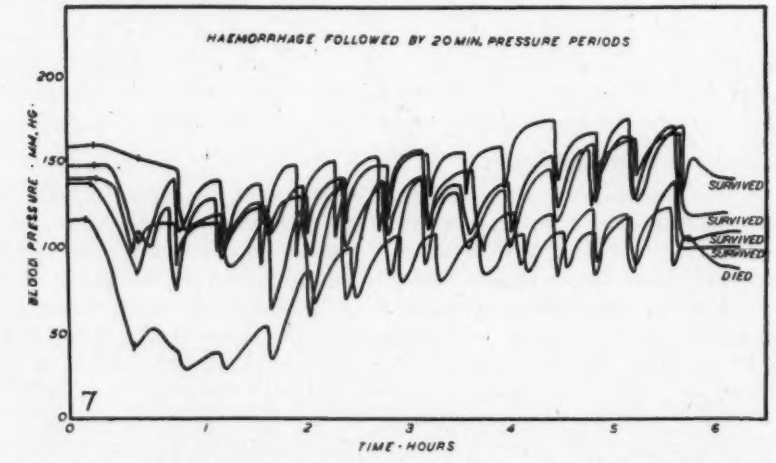
THE EFFECT OF INTERMITTENT PRESSURE PERIODS ON BLOOD PRESSURE WITH AND WITHOUT HAEMORRHAGE

Body weight, kgm.	Length of pressure periods, min.		Haemorrhage, cc.	Blood pressure				Result
	On	Off		Beginning	After haemorrhage	Lowest pressure on release of cuffs	After 6 hr. on final release of cuffs	
9.5	20	5	—	168	—	159	180	Survived 48 hr.
17.3	20	5	—	170	—	140	190	Survived 48 hr.
12.4	20	5	—	150	—	116	130	Survived 48 hr.
9.0	20	5	180	158	150	58	126	Survived 48 hr.
10.3	20	5	200	138	86	86	100	Survived 48 hr.
8.3	20	5	145	116	44	28	106	Survived 48 hr.
18.7	20	5	375	140	98	76	90	Died during first night
18.2	20	5	370	148	104	90	122	Survived 48 hr.
20.8	40	5	—	150	—	80	116	Survived 48 hr.
24.7	40	5	—	150	—	136	140	Survived 48 hr. but one leg very swollen
15.2	40	5	—	156	—	100	96	Died during first night
8.3	40	5	160	160	50	0	Died	Died 3 hr. later
14.0	40	5	280	138	56	56	68	Died during first night
6.2	40	5	105	146	64	24	40	Died during first night
15.0	40	5	300	150	34	34	60	Died during first night
19.6	40	5	395	168	154	96	104	Died during first night

Re-application of Tourniquets

It was observed in most of the early experiments with pressure cuffs that the blood pressure rose considerably after the pressure cuffs were applied (see tracing in Fig. 2, and Table I). Dogs were shocked by the method previously described and, after the blood pressure was reduced and the haematocrit values had increased, narrow band tourniquets were re-applied to the legs as tightly and as high up as possible. The blood pressure quickly rose. In some animals it was maintained at a high level for long periods of time despite the fact that the fluid lost into the injured limbs was still cut off from the body. In other dogs the pressure fell again later and the animals died. The changes in blood pressure in two of these animals are shown in Fig. 10. The ability to maintain this new level varied from dog to dog. In some the pressure stayed up for several hours with no signs of progressive shock. These animals became less cyanosed, the tongue became pink in colour again, and many dogs became so active that additional anaesthetic was required. In others the rise in pressure was just as great but was not maintained for a very long period. This may be due in part to the difficulty in shutting off all circulation to the leg by a narrow rope tourniquet, but a pneumatic cuff might be expected to force some fluid from the leg into the circulation and it was desired to avoid this if possible. It will be seen that the elevation in blood pressure can hardly be accounted for on the basis of restoration of blood volume since the fluid was trapped in the limbs. The haematocrit values



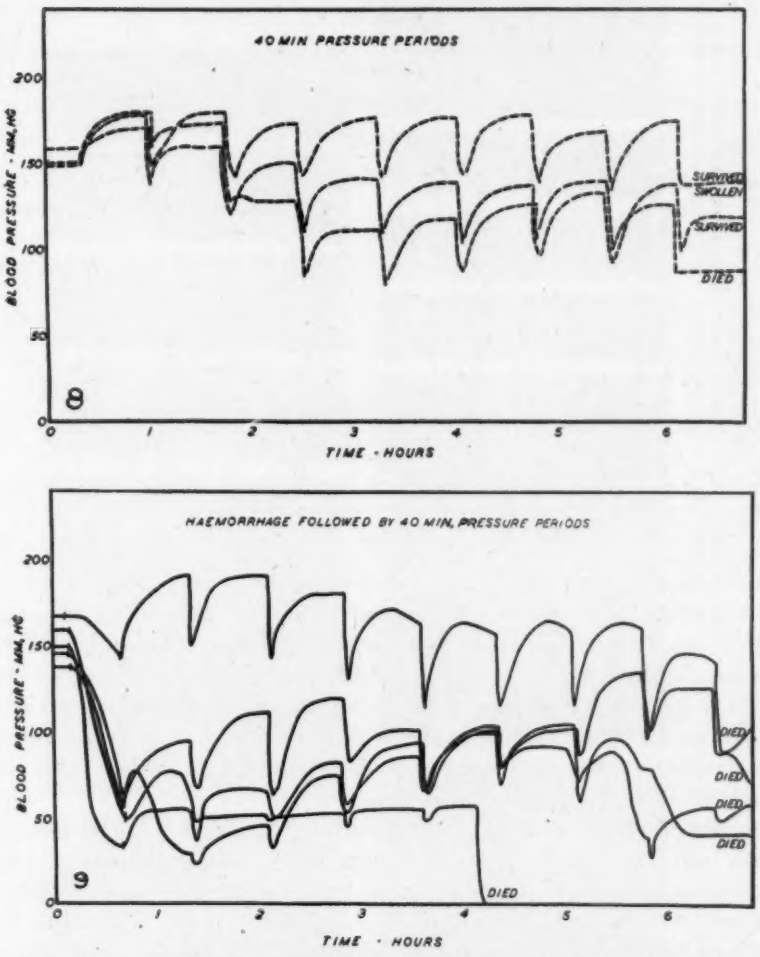


FIGS. 6 AND 7. *Blood pressure changes when cuffs are applied intermittently.* FIG. 6. *Twenty minute periods of application with five minute release intervals.* FIG. 7. *The same following haemorrhage (2% of body weight).*

were slightly reduced in those animals in which the new higher blood pressure was maintained. Where there was a subsequent fall in blood pressure, the haematocrit values continued to rise slightly. Table VII summarizes these data.

Compression of One Leg for a Longer Period

When cuffs were applied to both legs for the five hour period, most animals died following release of the cuffs. Urine flow ceased. It was desirable to



FIGS. 8 AND 9. *Blood pressure changes when cuffs are applied intermittently.* FIG. 8. *Forty minute periods of application with five minute release intervals.* FIG. 9. *The same following haemorrhage (2% of body weight).*

study animals that survived, so in a series of dogs one cuff was applied to one thigh for a five hour period.

In three dogs treated in this way there was no gross evidence of kidney damage. There was no albuminuria nor haematuria and the plasma non-protein nitrogen level did not increase.

In six dogs, when one cuff was applied to one thigh for 10 hr., the effect was more marked than after five hours, as indicated by limb swelling and haemoconcentration. These animals all survived the acute shock and were

TABLE VII

THE EFFECT OF RE-APPLICATION OF TOURNIQUETS TO THE HIND LEGS AFTER SHOCK HAS DEVELOPED

Weight, kgm.	Times				Blood pressures, mm. mercury				Haematocrits, % cells		
	Cuffs off, to tourniquets on		Tourniquets on, to termination		Before cuffs removed	When tourniquets applied	Highest after tourniquets on	Terminal	When cuffs removed	When tourniquets applied	Terminal
	Hr.	Min.	Hr.	Min.							
15.8	1	30	1	0	200	90	130	130	46.4	68.2	—
18.7	1	30	4	15	190	76	120	128	61.7	80.0	79.4
11.8	1	10	5	0	159	54	90	96	35.2	52.1	48.0
11.4	1	30	4	0	150	40	80	Died	30.1	56.2	55.5
5.7	0	25	4	0	170	18 art. resp.	110	Died	50.0	—	—
8.8	1	30	5	0	152	46	90	78	48.2	71.6	78.8
13.5	0	50	0	50	150	40	61	Died	68.5	80.9	—
12.2	1	55	0	50	174	70	81	Died	54.7	72.9	75.9
23.7	2	20	3	0	158	92	112	68	56.8	72.5	73.0
9.9	0	50	3	0	132	70	90	Died	53.4	62.0	64.0
13.5	2	0	3	0	210	73	134	134	56.4	81.0	75.8

observed for a period of four to six days before gangrene required their sacrifice. Within half an hour after removal of the cuff, grossly bloody urine was observed in all the dogs. With one exception this cleared up after 24 hr. Albuminuria persisted about three days. The daily volume of urine was large, with a low specific gravity and decreased chloride content. The plasma non-protein nitrogen rose about 90% or 20 mgm. % soon after removal of the cuff or on the following day, but, with one exception, was returning to normal on the second day (Fig. 11).

The animal noted above as an exception had oliguria, a continued rise in plasma non-protein nitrogen, a persistent albuminuria, and haematuria. There was a positive test for bile salts in the urine on the third and fourth days and the plasma was a brown colour. It was necessary to sacrifice this animal on the fourth day because of gangrene. This was the only animal that showed indications of persistent renal damage. The others showed only temporary signs of kidney change. The recovery period with falling plasma non-protein nitrogen and increased excretion of urine resembles that recorded for mild clinical cases of 'crush syndrome'.

The changes in limb volume and in haematocrit values in the dogs with one cuff applied for 10 hr. are interesting to compare with the changes in those dogs having two cuffs applied for five hours. Both showed roughly the same total increase in limb volume and similar increases in haematocrit, and yet the dogs with one cuff applied for 10 hr. survived, whereas the dogs with two cuffs applied for five hours died. These results are shown in Table VIII.

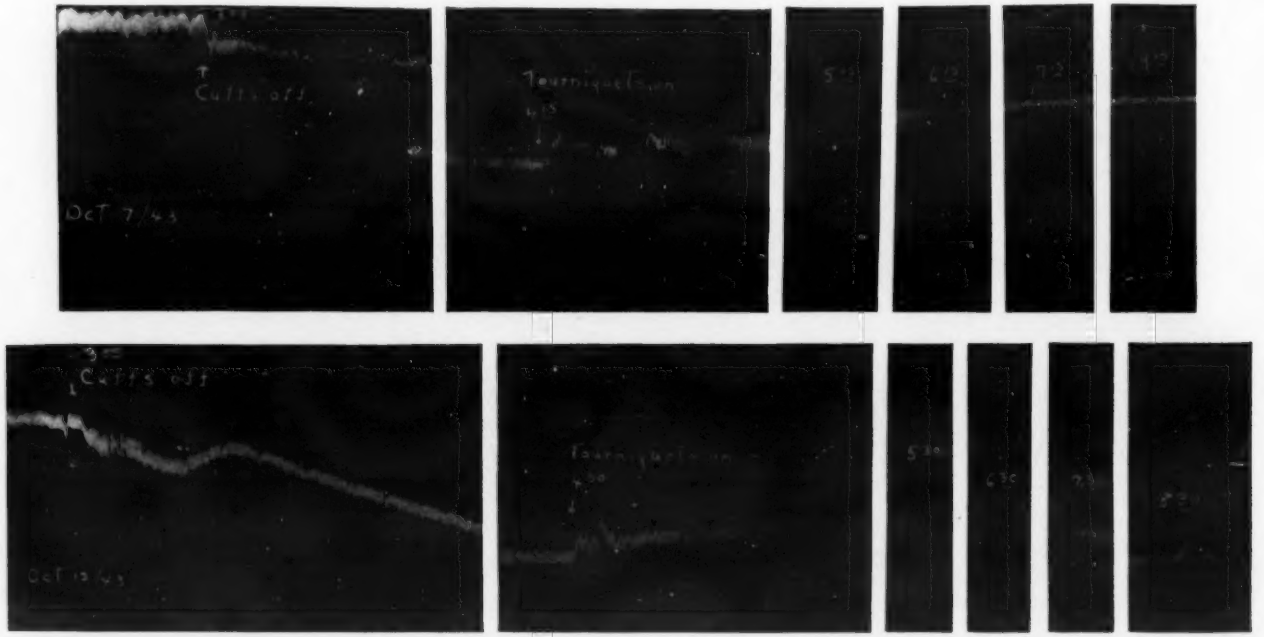


FIG. 10. *Blood pressure records showing the effect of re-application of narrow tourniquets after the limb-swelling had occurred. Upper record: mean pressure at beginning of tracing was 160 mm. mercury; at time of re-application was 54 mm. mercury; at end was 100 mm. mercury. Lower record: mean pressure at beginning was 140 mm. mercury; at time of re-application of tourniquet was 38 mm. mercury.*

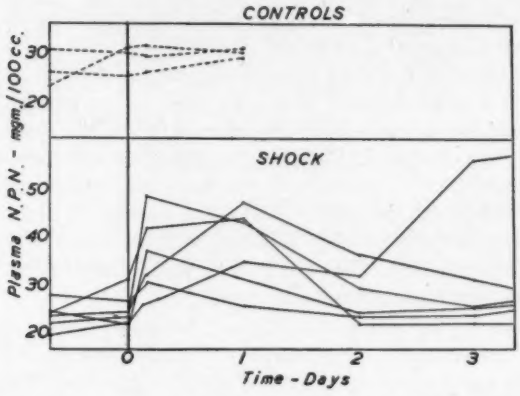


FIG. 11. *The changes in plasma non-protein nitrogen of control dogs and dogs surviving the application of one cuff for 10 hr.*

TABLE VIII

A COMPARISON OF FLUID LOSS AFTER APPLICATION OF A CUFF TO ONE LEG FOR 10 HR. AND TO BOTH LEGS FOR FIVE HOURS

Experimental procedure	Average increase in limb volume as % body weight	Average haematocrit late in shock	Average % increase in haematocrit after removing cuffs	Final result
Two cuffs for 5 hr. (22 dogs)	2.35	70.8	44	Fatal
One cuff for 10 hr. (6 dogs)	2.67	72.2	45	Survival

Discussion

Cannon and Bayliss (3) concluded as a result of both clinical and experimental investigations during the last war that a toxic substance was released from injured tissue and was the important factor in shock. This conclusion was questioned by Blalock (2) who claimed, as a result of weighing normal and injured limbs, that fluid loss at the site of the injury was sufficient to cause all of the symptoms of shock.

Using the volume displacement method, Solandt and Best (6) found that in traumatic shock produced by pounding the thigh muscles, 85% of their animals lost less than 50% of the calculated blood volume into the injured area. Slow bleeding indicated that 60% was necessary to produce death. They concluded that local fluid loss, while an important factor in initiating shock, was not always sufficient to cause death.

In the experiments reported here it is evident that the local loss of fluid was less than this and yet the animals died in a shorter time. It must be

borne in mind, however, that in 'pressure' shock, plasma is lost and the haematocrit value increases greatly, whereas in shock due to limb pounding considerable whole blood is lost and there is not much increase in haematocrit. The loss of such a large amount of plasma may be an important causative factor in many of the animals with this type of 'pressure' shock. However, when the injury was produced in one leg, the fluid loss and increase in haematocrit was as great as with damage to two legs, but when only one leg was involved the animals did not die. The rate of loss of fluid probably is important. There is the possibility too that with ischemia of a greater mass of peripheral tissue (muscle, skin, etc.), some factor in addition to fluid loss is present in sufficient strength to cause death.

In the experiments involving the application of the tourniquets fluid loss alone does not account for the changes in blood pressure. In nearly all instances the re-application of a narrow band tourniquet high up on the damaged limbs led to an increase in blood pressure (usually 40 mm. mercury or more). If the fluid already lost into the limb were responsible for the fall in blood pressure, then since the fluid remains lost to the body after the tourniquet is re-applied, the blood pressure should remain low. Since the blood pressure is elevated, some factor other than fluid loss must be introduced to account for this effect of the re-application of tourniquets on blood pressure. One possibility is that by cutting off a region in which vessels are dilated and the flow is slow, the blood in the remaining part can circulate more effectively using, as it were, a reduced circuit. Though the volume of blood in the remaining vascular bed is presumably the same as before the re-application of the tourniquets, a lesser cardiac output probably would be required to maintain the arterial blood pressure. A second possibility is that the re-application of the tourniquets stops the entrance of so-called 'toxic' materials from the limbs into the general circulation. A third possibility is that the strong sensory stimulation produced by the application of the tourniquets exerts a pressor effect.

The results of re-application of the tourniquets in the dog are comparable to the effect of reclamping in rats (4). Some survive but others do not. It would appear that a stage develops following which re-application of the tourniquets is only temporarily effective.

While fluid loss is the initiating factor in shock following haemorrhage, in certain other types of experimental shock fluid loss may not play as important a role. It should be remembered that it does not kill *per se* but must bring other changes that are responsible for death. If this be admitted, then the possibility must be granted that other factors besides fluid loss may bring about similar changes. Hence, shock may be *initiated* by a number of different events, but the fundamental changes that *characterize the state of shock* and are responsible for the fatal outcome probably are similar.

The effect of intermittent compression of the limbs is important since it gives information that may be of value in assessing the effects of the application of tourniquets in injured individuals. It will be evident from the data

presented that 20 min. periods of application of two cuffs do not lead to shock even when extensive bleeding has occurred, but that 40 min. periods of application may lead to a fatal outcome following haemorrhage in the dog. Even with the 20 min. periods of application, however, the fall in blood pressure after each release may be very definite.

Acknowledgments

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APPLICATION AND CONTROL OF ETHYL-ETHER–WATER INTERFACE EFFECTS TO THE SEPARATION OF RICKETTSIAE FROM YOLK SAC SUSPENSIONS[1]

By James Craigie[2]

Abstract

Purified suspensions of rickettsiae may be obtained by shaking impure suspensions with ethyl ether. When such mixtures separate, tissue particles remain at the interface and the organisms are found in the underlying aqueous fraction. If crude yolk sac preparations of rickettsiae, grown by the method of Cox (4), are shaken with ethyl ether, a complex physical system is created, and the results obtained may be variable. In order that ether treatment may yield sufficiently consistent results to be of practical value, it is necessary to control certain physical factors during emulsion formation and subsequent separation of the aqueous phase. The result obtained depends mainly on the initial hydrogen ion concentration of the suspension, although other factors may also be involved. Studies of some of these factors have been undertaken and two methods of purifying rickettsiae have been developed. In Method *A*, the material is centrifuged and the sediment of rickettsiae, tissue debris, and yolk granules is suspended in saline buffered with phosphate at pH 7.0. This partially purified suspension of rickettsiae is then emulsified with ethyl ether, and the rickettsiae are recovered in the aqueous fraction that separates as the unstable emulsion breaks down.

Preliminary centrifugation is dispensed with in Method *B*. The separation of the aqueous fraction, with maximum yield of rickettsiae, is controlled by the addition of a suitable proportion of acetate buffer to the crude yolk sac suspension. The optimum proportion of acetate buffer is determined in a preliminary titration with small samples of the yolk sac suspension.

The usefulness of the ethyl-ether–water interface in promoting separation of rickettsiae from the tissue in which they have been cultivated depends on the fact that rickettsiae in common with a number of viruses (e.g., poliomyelitis and vaccinia virus) and bacteria (e.g., *Salmonella* species, staphylococci, streptococci, pneumococci, etc.) are repelled from the interface, while insoluble tissue or medium constituents are selectively attracted to the interface. For this purpose, ethyl ether is definitely superior to other substances such as petroleum ether, and this is probably related to the relatively greater solubility of the former in water. Ethyl ether has the additional advantage of being bactericidal and capable of rendering rickettsiae non-infective with great rapidity.

When ethyl ether is applied to the processing of virus-infected tissue extracts that are relatively deficient in lipoids and emulsifying substances, the interface area obtained is variable and limited, necessitating the addition of substances that permit its extension by emulsion formation, either by

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Contribution from the Connaught Laboratories, University of Toronto, Toronto, Ont., with financial assistance from the National Research Council of Canada.

[2] *Research Member, Connaught Laboratories, and Associate Professor of Virus Infections, School of Hygiene, University of Toronto, Toronto, Ont.*

lowering interfacial tension or providing conditions for the formation of a film of sufficient mechanical strength around the disperse phase. On the other hand, yolk sac suspensions are rich in emulsifying agents, and the formation of too extensive and permanent an emulsion is likely to occur under certain conditions, with the result that separation of the continuous aqueous phase is prevented or delayed. Delay in separation of the continuous aqueous phase involving prolonged contact with ether (4 to 24 hr.) prejudices separation of pure suspensions of rickettsiae since: (*a*) elution of ether-soluble substances from tissue fragments may occur, with the result that they remain in the aqueous portion of the system; (*b*) some soluble proteins may become insoluble as a result of the prolonged exposure to ether.

The successful application of the selective effect of the ethyl-ether–water interface to virus-infected tissue extracts, and particularly to yolk sac suspensions, necessitates control of the extremely complex physical system involved. Not only must conditions be so arranged that all yolk sac tissue fragments and yolk granules are attracted to the interface while the rickettsiae are repelled, but a proper balance must be struck between (*a*) the initial development of a sufficiently extensive interface for segregation, and (*b*) the recovery of a sufficient volume of the continuous aqueous phase containing the rickettsiae to make the process of practical value.

Control may be exercised in the following ways:

1. By reducing the yolk content of the material to be processed to a point where the extent and stability of emulsion formation is kept within desirable limits (e.g., by preliminary high speed centrifugation);

2. By varying hydrogen ion concentration, which in turn affects emulsion development and separation, either by direct interface effect or by indirect effect on some of the constituents of the crude suspension;

3. By varying the nature and the concentration of the electrolytes present. This, as in the case of variation of hydrogen ion concentration, may produce effects directly or indirectly;

4. By adding water-soluble substances that raise or depress interfacial tension;

5. By altering the mutual solubilities of ethyl ether and water (*a*) by adding substances soluble in ethyl ether but not in water, or (*b*) by adding substances soluble both in ethyl ether and in water.

Obviously, studies in which these factors are varied independently constitute an essential preliminary to controlled application of interface effects to virus separations. In the case of rickettsia infected yolk sac suspension, several theoretical solutions are possible on the basis of ascertained data.

Method A

For a period of more than a year* the principles of this method have been used as a routine in the preparation of purified suspensions of murine and epidemic typhus rickettsiae. The method yields stable suspensions of

* *Prior to February, 1942.*

epidemic or murine rickettsiae that effectively protect guinea pigs against considerable multiples of the minimum infective dose of infective rickettsiae.

At the time of collection, the infected yolk sacs are allowed to drain on bronze screens but no attempt is made to wash adhering yolk from them. The yolk sacs are triturated by shaking in heavy glass bottles with glass beads, and the following diluent is used in the ratio of 5 or 10 cc. per yolk sac:

0.85% Sodium chloride in distilled water	4 parts
Sorensen's sodium potassium phosphate buffer, pH 7.0	1 part
Formalin (40% formaldehyde)	to 0.5%

The suspension is filtered through bronze gauze and placed in the cold room to permit the yolk cream to rise to the surface. The suspension is siphoned off from below the yolk layer and centrifuged at 4000 r.p.m. in the refrigerator to deposit the rickettsiae along with other particulate matter present. The yolk cake is carefully removed and the supernatant is discarded. Care is taken to swab adhering traces of yolk from the tube before proceeding to resuspend the deposit. The deposit is resuspended in diluting fluid similar in composition to that noted above. The diluting fluid is added in the proportion of 10 cc. per yolk sac represented by the deposit. The deposit, after thorough dispersion, is kept in the refrigerator for several days to permit sedimentation of coarse particles to occur. The supernatant, which contains rickettsiae in suspension, is processed thus:

A separatory funnel of suitable capacity is half-filled with the suspension and a half-volume of ethyl ether is added. The funnel is shaken vigorously and extensive emulsion formation occurs. If separation of ether at the upper surface does not begin within a few minutes, a further amount of ethyl ether should be added and incorporated by light shaking. In a short period on standing, provided care has been taken to exclude the light fraction of yolk, separation occurs into three layers: (*a*) an upper layer of excess ether, (*b*) a middle emulsion layer, and (*c*) a lower aqueous layer. The middle emulsion layer (*b*) contains tissue fragments and yolk particles while the lower aqueous layer (*c*) contains rickettsiae in suspension along with a reduced amount of cell debris.

The lower aqueous layer containing the rickettsiae is run off from the separatory funnel into a second separatory funnel. A further amount of ethyl ether is added, approximately one-fifth volume. The new mixture is shaken vigorously and then allowed to stand in the refrigerator for at least 30 to 60 min. Rapid separation into layers occurs on the second ether treatment, since emulsifying substances have been removed in the first treatment with ether. Consequently the interface area is limited to the line of separation and it is necessary to wait for a short period to permit remaining tissue fragments to slowly rise to the interface. When residual tissue fragments have migrated upwards to the interface the lower aqueous portion is run off into a third separatory funnel.

If proper care is taken to exclude the light fraction of yolk from the suspension being processed and the operator has sufficient experience of the procedure,

two treatments with ether may be sufficient to provide suspensions of rickettsiae of a high degree of purity. Generally, however, it is necessary to resort to a third treatment with ether. In this case, the aqueous portion from the second separatory funnel is run off into a third funnel and a one-fifth volume of ether is added. After shaking, rapid separation occurs, the separation on this occasion being characterized by the transient persistence, in the ether immediately above the interface, of large droplets of water that are surrounded by a film of tissue detritus.

With experience, the progress of the purification can be accurately judged by the opalescence and optical qualities of the aqueous fraction. Pure suspensions of rickettsiae exhibit, by reflected light, an opalescence that contrasts with their relative transparency when they are viewed by transmitted light. If viewed by transmitted light from a single artificial source, a pure suspension exhibits a faint yellowish orange colour, which contrasts with the faint bluish white opacity seen when the suspension is viewed by reflected light. In brief, the optical qualities are similar to those of pure vaccinia elementary-body suspensions.

It is not necessary, and is in fact undesirable, that the processing be continued beyond three treatments with ether. As mentioned above, prolonged exposure to ether apparently results in the elution of ether-soluble substances from the tissue fragments and instead of being attracted to the interface they are repelled into the aqueous portion. If a complete purification is not effected by three treatments with ether, further purification may be achieved by permitting the unwanted particles to sediment slowly from the suspension in the cold room in tall glass cylinders.

It is not necessary to resort to exposure to a partial vacuum to remove the dissolved ether present. The ether-treated suspension is stored in the cold room in glass cylinders covered with a light, sterile gauze, cotton-wool pad, which does not impede evaporation of the ether. If desired, the suspensions obtained by this method may be concentrated by a second high-speed centrifugation at 4000 r.p.m. If lusteroid tubes are employed, centrifugation should not be carried out until several days have been allowed for evaporation of dissolved ether, since lusteroid tubes become temporarily softened by exposure to ethyl ether. With a little experience, consistent success can be obtained in using this three-stage method of ether processing. It will be noted that the processing is carried out at pH 7.0 in the presence of a relatively strong concentration of phosphate buffer. Although a considerable proportion of ethyl ether is required, a commercial grade has been found quite suitable and a considerable quantity may be recovered for redistillation, particularly if the emulsions are partly broken with acid buffer.

Method B

This method depends on the effect of hydrogen ion concentration and choice and concentration of electrolytes on emulsion formation and contraction, and it permits the processing of crude yolk sac suspensions in one operation without

resort to preliminary centrifugation. Even on centrifugation at 4000 r.p.m., it is not possible to sediment all the smaller rickettsiae that may be present in large numbers and yet be difficult to detect in yolk sac impression smears. Application of ether treatment to uncentrifuged yolk suspension therefore eliminates this particular source of loss. The crude yolk sac suspension is prepared for Method *B* in the same way as for Method *A* except that formol saline solution without added buffer is employed. For processing, the yolk sac suspension is siphoned off from below the top layer of yolk cream. It is desirable to exclude the lighter fraction of yolk as far as possible, otherwise a greater proportion of buffer solution may be required.

The effect of hydrogen ion concentration and buffer concentration on emulsion formation and separation is illustrated diagrammatically in the following figures.

The Effect of Hydrogen Ion Concentration on Emulsion Separation

Three different batches of yolk sac suspension, all infected with the same Madrid strain inoculum, but collected in different pools, were tested. Two cubic centimetres of yolk sac suspension was placed in each tube of the series and 0.25 cc. of McIlvaine citric acid–phosphate buffer at the hydrogen ion concentration stated was added. Two cubic centimetres of ethyl ether was added then to each tube and each tube was subjected to 10 vigorous shakes. The readings for each series were taken 15 min. after shaking (Fig. 1*a*). Although separation progressed for one hour after shaking, no separation was noted in tubes that were negative at the 15-min. interval.

Fig. 1*b* shows diagrammatically the appearance after 12 hr. The late flocculation of protein that occurred between pH 4.3 and 5.9 should be noted.

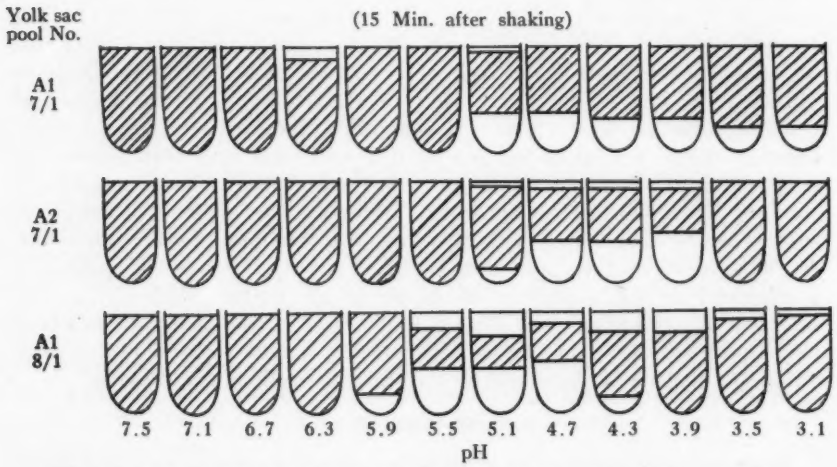

FIG. 1*a*. *Influence of hydrogen ion concentration on separation of ether–yolk-sac emulsions. Shaded portion—emulsion. Upper layer (where present)—separated ether. Lower layer (where present)—separated aqueous fraction containing rickettsiae.*

This is in part due to the prolonged exposure to ether at these hydrogen ion concentrations. Solution of yolk pigment in the ether on the acid side of pH 6.0 is not shown in Fig. 1*b*.

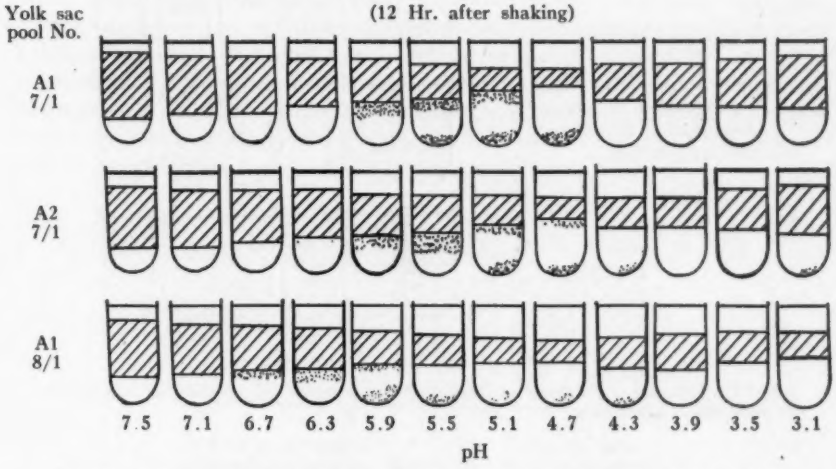

FIG. 1*b*. *Influence of hydrogen ion concentration on separation of ether–yolk-sac emulsions. Shaded portion—emulsion. Upper layer (where present)—separated ether. Lower layer (where present)—separated aqueous fraction containing rickettsiae. The dots represent protein precipitate and aggregation of cell debris.*

The Effect of Hydrogen Ion Concentration and Concentration of Buffer on Emulsion Formation and Separation

This is illustrated in Fig. 2. The buffer used was again citric acid phosphate buffer and the volumes of yolk sac suspension and ethyl ether were 2 cc. of each per tube. Each tube was subjected to only four complete shakes, and it may be pointed out here that under optimum conditions for any particular yolk sac suspension, variation in the amount of shaking has relatively little effect on the degree of separation and purification. This, however, is not the case under suboptimal conditions.

As shown in Fig. 2, the separation of the yolk–ether emulsion is dependent on the acidity and concentration of the buffer solution. As the acidity of the buffer is increased, smaller quantities of buffer are required to produce the desired effect.

Antagonistic Action of Sodium Chloride

As shown in Fig. 3, the concentration of sodium chloride present affects the result obtained, and its antagonistic action is possibly related to its capacity to hold protein in solution.

Comparison of Buffer Systems

A number of buffer systems have been compared. It has been found that sodium acetate–acetic acid buffer, on the basis of molar concentration, is

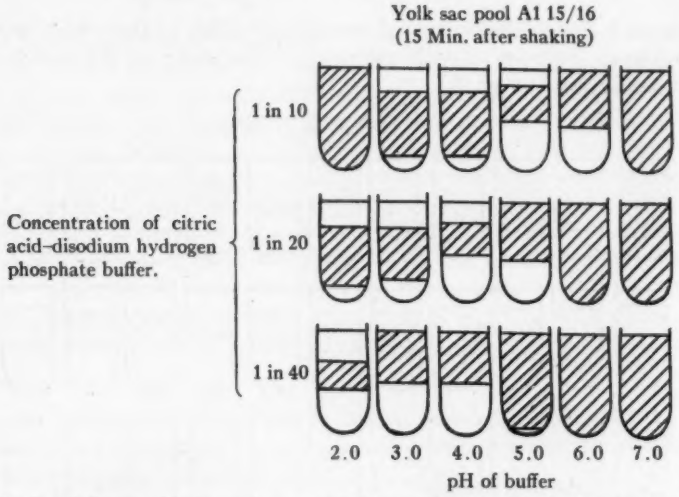

FIG 2. *Influence of hydrogen ion concentration and buffer concentration on separation of an ether–yolk-sac emulsion.*

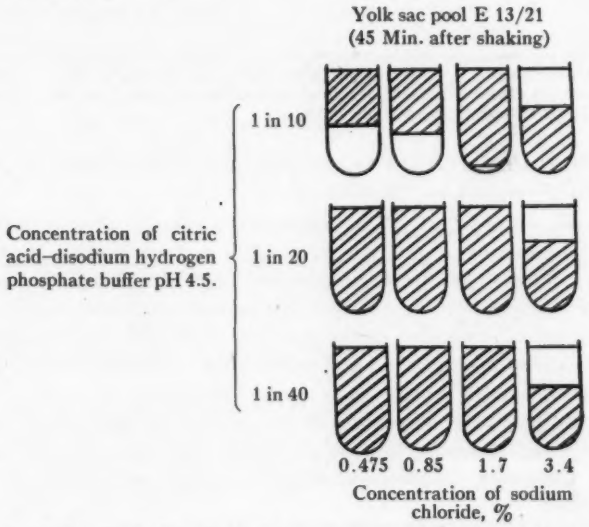

FIG. 3. *Influence of salt concentration on separation of an ether–yolk-sac emulsion.*

more effective than citric acid–phosphate buffer or sodium citrate–hydrochloric acid buffer. An added advantage of the acetate buffer lies in the fact that the region of equimolecular amounts of sodium acetate and acetic acid provide a suitable hydrogen ion concentration for processing and that later adjustments of hydrogen ion concentration to a more acid reaction can readily be obtained by the addition of citric acid.

Determination of Amount of Buffer Required for Processing

As is to be expected, minor variations in the composition of yolk sac suspensions, which are dependent upon (*a*) the age of the eggs at the time of death of the embryo; (*b*) extent of infection of the yolk sac with rickettsiae; (*c*) variations in the effectiveness of trituration of the yolk sacs; and (*d*) time of storage, produce variations in the optimum amount of buffer required for controlled separation. These variations, however, present no obstacle from the practical point of view since the optimum concentration of buffer can be quickly determined by means of a preliminary test carried out on a sample of the yolk sac pool. An example of such a titration is illustrated in Fig. 4.

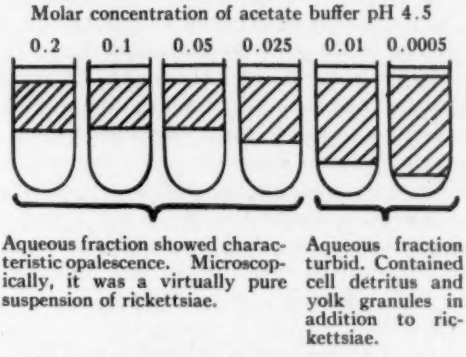

FIG. 4. *Titration of molar concentration of acetate buffer pH 4.5 required for ether processing of yolk sac pool ABC 13/16.*

It has been found that the optimum hydrogen ion concentration of acetate buffer lies between pH 4.4 and 4.6. The physical reactions that occur at this hydrogen ion concentration are more complicated than in Method *A* since certain proteins in solution are precipitated out and adsorbed at the ethyl-ether–water interface. It is undesirable to employ a more acid buffer because, although smaller quantities are required, the hydrogen ion concentration approaches too closely that of the isoelectric point of the rickettsiae, and consequently some loss may occur. In practice, Method *B*, which is at present* on trial, is carried out as follows.

Details of Method B

Yolk sac suspension in formol saline, without added buffer, is allowed to stand for several days in the cold room to permit sedimentation of coarse particles and separation of yolk cream, which rises to the top. The suspension is carefully siphoned out in such a way as to leave the deposit undisturbed and leave all traces of yolk cream behind. A small volume of the suspension is set aside for preliminary titration and the volume of the remainder is determined. The titration is carried out by distributing a series of volumes of

* *I.e., February, 1942.*

molar or 1/10 molar acetic acid–acetate buffer in a series of tubes. A suitable volume of yolk sac suspension (2 cc.) is added to each tube. Two cubic centimetres of ethyl ether is then added and each tube is subjected to six vigorous complete shakes. The tubes are kept under observation and the order in which breaking of the emulsion occurs is noted. The concentration of buffer required is judged (*a*) by the rate of separation, and (*b*) by the optical qualities of the bottom aqueous portion (see Fig. 4). If insufficient buffer is present, fairly rapid separation may occur but the aqueous portion shows greater density and the optical qualities suggest that it is impure and microscopic examination will show that cell debris and flocculent proteinaceous material have not been completely eliminated. With experience, the optimum concentration of buffer can be determined within 15 min. of setting up the titration. The volume of the yolk sac suspension being known, the desired amount of acetate buffer is calculated and added. An equal volume of ethyl ether is then added and the mixture is shaken briefly. It should be noted that it is not necessary to resort to such extensive shaking as in Method *A*. The emulsification with ether is conveniently carried out in screw-cap, four-litre bottles. When separation of the aqueous portion begins, a siphon system is inserted, leading to the bottom of the bottle, and any fluid that has crept into the siphon tube is expelled by positive pressure. Separation is allowed to proceed in the cold room for 30 to 60 min., when the aqueous fraction is siphoned off. This is transferred to open-top cylinders that are covered with a light gauze and cotton wool protective cover and allowed to stand for a day or two for evaporation of ether before the contents is centrifuged. Centrifugation may be carried out either at pH 4.4 to 4.6 with a more effective recovery of rickettsiae or the hydrogen ion concentration may be adjusted to pH 7.0 immediately after the aqueous fraction has been separated. Buffer saline should be employed in resuspending the deposit, particularly when the centrifugation is carried out at pH 4.4 to 4.6. Should microscopic examination of the resuspended deposit show incomplete purification owing to error in the adjustment of the amount of acid buffer employed or insufficient shaking with ether, complete purification can be obtained by shaking the resuspended deposit with ether at pH 7.0.

Studies on the antigenicity of rickettsiae obtained by Method *B* are in progress. The yield obtained by Method *B* appears to be greater than that obtained by Method *A*. Method *B* has also the great advantage of eliminating one centrifugation and reducing ether processing to one treatment instead of the three called for in Method *A*. Other investigations are in progress relative to the possibilities of still more simplified methods.

Supplementary Note

Ethyl ether has been used in various ways by a number of investigators in the virus field. In reviewing these applications of ethyl ether, it should be borne in mind that this substance has three distinct and useful properties that may be exploited singly or in combination. It has been used as a bac-

tericidal agent, as a lipoid solvent, and as a means of removing impurities from virus suspensions.

The majority of bacterial species are rapidly killed by exposure to ethyl ether in saturated solution in an aqueous medium, but many viruses are resistant to this treatment. Vincent (15) compared the immunizing qualities of suspensions of *Bacterium typhosum* that had been killed in various ways and concluded that an ether-treated vaccine was to be preferred. Fornet (8) appears to have been influenced by Vincent's observations and in 1913 he reported experiments in which fresh calf lymph was shaken with ethyl ether for periods of 20 hr. or more. He found that vaccine lymph was usually freed from all bacterial contamination by such treatment, although the potency of the virus was in no way affected. In 1917 Amoss and Taylor (1) and Taylor and Amoss (13), in their investigations of nasopharyngeal secretions, pointed out that poliomyelitis virus was resistant to ethyl ether, and they used ether for its bactericidal qualities. Subsequently ether was used by other investigators, for example, by Kramer, Sobel, Grossman, and Hoskwith (11) in 1936 in testing nasopharyngeal secretions for poliomyelitis virus. When attention was redirected to the occurrence of this virus in the human gastro-intestinal tract, ethyl ether was used to eliminate infective bacteria from stool specimens by Trask, Vignec, and Paul (14), and by Kramer, Hoskwith, and Grossman (10).

The fat-solvent qualities of ethyl ether have been exploited in the investigation of the *in vitro* serological reactions of viruses. Craigie and Tulloch (6), in their work on the variola-vaccinia flocculation reaction, employed ether primarily as a lipoid solvent in preparing antigen extracts from smallpox and vaccinia lesions. This use of ether was suggested by the observation that extracts of rabbit brain had an inhibitory effect on the flocculation reaction (Burgess, Craigie, and Tulloch (2)). Howitt (9) employed ether in this way in preparation of brain antigens in her work on the complement-fixation reactions of equine encephalomyelitis and lymphocytic choriomeningitis.

The third useful property of ethyl ether is its capacity to form emulsions that may be used to remove, selectively, tissue impurities from crude virus suspensions. Ledingham (12) in 1931 employed ether in the preparation of vaccinia and fowl-pox elementary bodies for agglutination tests. He triturated tissue infected with these viruses in distilled water, added an equal volume of ether, and shook the mixture at intervals for 24 hr. At the end of this treatment, he centrifuged the mixture at high speed. This author describes separation of the centrifuged mixture into an "upper layer of ether, a large middle layer of light fatty matter" and an "underlying opalescent fluid". Craigie (5), however, did not obtain consistent results with this technique and concluded that ethyl ether was to be avoided in the preparation of purified suspensions of vaccinia elementary bodies. In a later modification of technique, Craigie and Wishart (7) added an excess of ether after the virus had been separated by centrifugation, and shook the mixture thoroughly. The preparations were stored in the cold room and the purified virus was removed

from under the overlying ether as required. Clark, Rasmussen, and White (3) have reported on the use of ethyl ether in the purification of poliomyelitis virus. They describe the effect of shaking saline suspensions of infected monkey cord and brain with ethyl ether. They state that three layers were commonly present when the mixtures of ether and saline suspensions of monkey cord were permitted to separate, viz.: an upper ether layer, a middle proteinaceous layer containing little virus and a lower watery layer containing most of the virus. Similar observations were made by Craigie and Fisher* in investigations relating to the isolation of poliomyelitis virus from stool specimens.

The initial work by the present author in which ethyl ether was employed in preparing suspensions of poliomyelitis virus or rickettsiae clearly demonstrated that the results might vary considerably. Separation or breaking of the emulsion frequently failed to occur when attempts were made to purify the crude preparations with ethyl ether. When the method was applied to crude yolk sac suspensions of rickettsiae, marked variations in the yield of purified organisms became evident even when separation of the emulsion appeared to be satisfactory. It therefore became obvious that some further study was required if the method was to be brought under suitable control for the preparation of typhus vaccine. This was undertaken and Methods *A* and *B* were devised. Method *A* remains the method of choice for the small-scale preparation of pure suspensions of rickettsiae for serological investigation. In typhus vaccine production, Method *B* was subsequently replaced by a method of processing crude yolk sac preparations of rickettsiae at pH 7.0.

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